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ADENYLATE CYCLASE ASSAY WITH ADENYLYL IMIDODIPHOSPHATE AND PRODUCT DETECTION BY COMPETITIVE PROTEIN BINDING

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SUMMARY

An assay for adenylate cyclase (EC 4.6.1.1) is described using adenylyl imidodiphosphate (AMP–PNP) as substrate. Methods are described for introducing the cyclase reaction mixture directly into a protein binding assay for the cyclic nucleotide without purification of cyclic AMP. Significant substrate depletion and regenerating systems are avoided by this method, and blank values are negligible. The assay is capable of reproducibly detecting adenylate cyclase activity with less than 5 μg of protein from rat cerebral cortex.

INTRODUCTION

The assay of adenylate cyclase (EC 4.6.1.1) is complicated by the necessity for determination of low enzymatic activities (pmoles/mg protein per min) in the presence of competing reactions with activities several orders of magnitude greater. While much valuable data has been obtained with the method of Krishna and coworkers [1, 2], this method is somewhat limited by sensitivity and, when [^{3}H]ATP is used as the substrate, by specificity. Rodbell et al. [3] recently have used an isotopically labeled analog of ATP, adenylyl imidodiphosphate (AMP–PNP) as a substrate in the assay described by Krishna et al. [1]. The replacement, by nitrogen, of the oxygen between the β- and γ-phosphates renders the molecule essentially immune to enzymatic cleavage at the β,γ-position by ATPase and other phosphohydrolases [4]. However, while AMP–PNP was shown to be a substrate for adenylate cyclase, no data have been published characterizing the reaction of AMP–PNP with the enzyme, e.g. pH optimum, K_m, V.

The utilization of labeled AMP–PNP of course necessitates the separation of substrate and product. If an assay of high sensitivity is required, this can be a formidable task, since a 10^4–10^5-fold excess of substrate must be removed. The protein binding assay for cyclic AMP [5] has this degree of specificity and eliminates the need

Abbreviations: SQ-20009, 1-ethyl-4-(isopropylidenehydrazino)-1*H*-pyrazolo-(3,4-b)-pyridine-5-carboxylic acid, ethyl ester; RO 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

to use μCi/tube amounts of [^{32}P]AMP–PNP. Accordingly, we have characterized the reaction of adenylate cyclase from rat cerebral cortex with AMP–PNP and have developed simple methods for quantification of unlabeled cyclic AMP produced in reaction mixtures containing AMP–PNP via the protein binding assay. This procedure is capable of determination of the enzymatic activity of less than 5 μg of protein from rat cerebral cortex.

MATERIALS AND METHODS

Materials

AMP–PNP was purchased from International Chemical and Nuclear Corp., Irvine, Calif. in reported purities of 87–92%. However, chromatography (see below) indicated purities of only 70–90%, the major contaminant being 5′-adenylyl phosphoramidate (ADP–NH_2). Preliminary data indicate that this compound may influence enzymatic activity. Some lots were also contaminated with approximately one part in 10^4 cyclic AMP as determined by chromatography on Dowex-1-Cl paper, and by cyclic 3′,5′-nucleotide phosphodiesterase (phosphodiesterase, EC 3.1.4.1). To use available preparations of this substrate it is thus essential to purify the AMP–PNP, and an adaptation of the procedure of Yount et al. [4] was utilized. Triethylammonium bicarbonate was prepared by bubbling CO_2 through a solution of 140 ml of triethylamine and 500 ml water at 0 °C until the pH fell to 7.5. Subsequent dilution to 1 l gave a 1.0 M stock solution. A 2.5 cm × 30 cm DEAE column was equilibrated with 10 mM triethylammonium bicarbonate in the cold and 150 mg of AMP–PNP dissolved in the same buffer was added to the column. ADP–NH_2 (and cyclic AMP) was eluted with 100 mM triethylammonium bicarbonate, while the AMP–PNP was eluted with 200 mM buffer. The eluate volume was reduced to 20 ml on a rotary evaporator at room temperature. About 50 ml of methanol was added, and the solution taken to dryness. After redissolving in a small amount of water, more methanol was added, and the evaporation repeated twice more. The final dried AMP–PNP was redissolved in cold water to the desired concentration and stored as a stock solution at −20 °C. The purified AMP–PNP migrated as a single spot in one electrophoretic and two paper chromatographic systems: (a) Whatman No. 31ET (descending), 1-propanol–conc. NH_4OH–water (6:3:1, v/v/v); (b) Whatman No. 1 (descending), 95% ethanol–0.5 M ammonium acetate (5:2, v/v); (c) electrophoresis on Whatman No. 3MM, 30 min, 3 kV, pH 3.5, in pyridine–acetic acid–water (5:50:945, v/v/v). We have recently learned that AMP–PNP may also be obtained from P–L Biochemicals or Boeringher–Mannheim although we have had no experience to date with these preparations.

All other chemicals were purchased from standard sources in the highest available grade. Inhibitors of phosphodiesterase were obtained as follows: SQ-20009 [1-ethyl-4-(isopropylidenehydrazino-1*H*-pyrazolo-(3,4-b)-pyridine-5-carboxylic acid, ethyl ester] from Squibb and Sons; 1-methyl-3-isobutylxanthine from Dr R. L. Bergstrom, Searle Laboratories or from Aldrich; RO 20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone] from Dr H. Sheppard, Hoffman–La Roche.

Enzyme preparation

Crude adenylate cyclase was prepared from 60-day rat cerebral cortex by centrifugation of a 10% (w/v) homogenate in 0.32 M sucrose for 300 000 × g × min.

The pellet was resuspended in approx. 10 vol. of 5 mM Tris–Cl, pH 7.5, homogenized, and centrifuged as above. This washed pellet was resuspended at 3–5 mg protein/ml in 5 mM Tris–Cl, pH 7.5 and frozen in small aliquots at −90 °C until use.

Enzyme assay

The standard adenylate cyclase assay contained, in a total volume of 100 μl, the following concentrations of materials: 20 mM Tris–Cl, pH 8.5 (30 °C), 10 mM NaF, 2 mM $MgSO_4$, 1 mM theophylline or 0.1–0.2 mM 1-methyl-3-isobutylxanthine, 0.5 mM AMP–PNP and 5–50 μg of enzyme protein. Bovine serum albumin (10 μg) was added as noted. Reactions were initiated by addition of enzyme and were stopped, after incubation for 10 min at 30 °C, by the addition of sodium acetate–acetic acid, pH 3.9 (final pH 4.1) to a final concentration of 50 mM or by the addition of 50 μl of 0.3 M HCl (final pH 1.0–1.1). Samples with HCl were heated at 90 °C for 30–60 min on a heat block, followed by lyophilization and resuspension in 50 mM sodium acetate, pH 4.5. If large quantities of cyclic AMP (> 20 pmoles) are produced, final volumes should be 500 μl or greater (see below). When ATP was used as a substrate, the pH of the reaction mixture was 7.5 and [Mg^{2+}] was 4 mM. Samples were routinely frozen at - 20 °C until assay for cyclic AMP. Protein was determined by the method of Lowry et al. [6].

Cyclic AMP assay

Cyclic AMP was measured [5] in a final volume of 50 μl containing 50 mM sodium acetate–acetic acid, pH 4.0, 1.0 μmole cyclic [^{3}H]AMP, 10 μg protein kinase inhibitor, up to 20 μl of sample, and sufficient binding protein (1 μg) to bind approx. 30% of the cyclic [^{3}H]AMP. Binding protein was prepared from bovine skeletal muscle as described [5] and diluted before use with bovine serum albumin sufficient to add 10 μg/tube. After at least 60 min at 0 °C, the samples were diluted with cold 20 mM potassium phosphate pH 6.0, immediately filtered through Millipore HAWP filters, and washed with 8 ml of potassium phosphate buffer before being dissolved for counting in 3:1 toluene–methyl cellosolve containing 4 g/l diphenyloxazole.

RESULTS

Adenylate cyclase reaction conditions

The pH dependence of adenylate cyclase with AMP–PNP as a substrate is somewhat distinct from that seen and reported with ATP. NaF-stimulated adenylate cyclase exhibits a broad pH optimum with a peak below pH 8 when ATP is the substrate [7], whereas with AMP–PNP, an optimum at about 8.5–8.8 is obtained (Fig. 1). This shift in pH optimum has also been observed with basal and NaF-stimulated adenylate cyclase activity from guinea pig heart and two cultured cell lines (Wiklund, R.A., Maguire, M. E. and Gilman, A. G., unpublished observations). Similar results were obtained with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) buffer. This pH shift might be due to the higher pK_a of the γ-phosphate in AMP–PNP compared to ATP: 7.7 vs 7.1, respectively [4]. The higher pH does not seem to affect the enzyme adversely, since cyclic AMP production is linear with protein to 60 μg (Fig. 2) and with time for at least 15 min at 30 °C (Fig. 3). The reaction does not, however, appear to be linear for even 5 min at 37 °C with or without added bovine serum

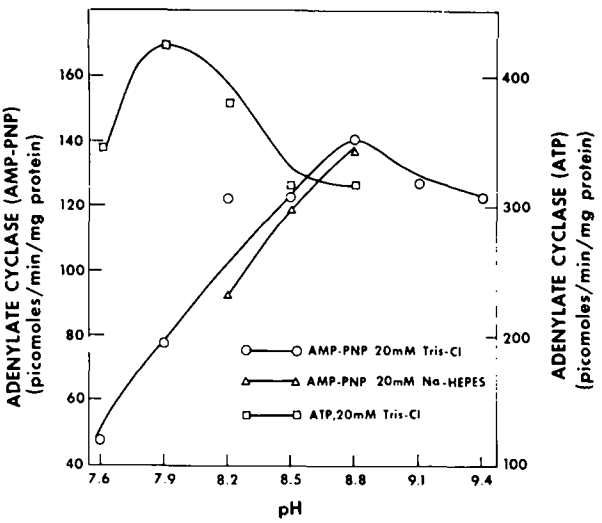

Fig. 1. Effect of pH on adenylate cyclase activity. □--□, ATP (2 mM), Tris-Cl (20 mM); ○—○, AMP-PNP (0.5 mM), Tris-Cl (20 mM); △—△, AMP-PNP (0.5 mM), Na-HEPES (20 mM).

albumin. Addition of bovine serum albumin at 30 °C enhances cyclic AMP production approx. 20–30% but does not prolong the period of linearity.

Because of depletion of ATP during the reaction, it is difficult to obtain satisfactory kinetic data without use of an ATP-regenerating system. However, it was possible to determine that the rat cortical preparation used exhibited a maximum activity approximating 200–400 pmoles/min per mg protein, in reasonable agreement with other data reported [8–10]. Although determinations of the K_m for ATP were not completely satisfactory, values approximating 50–100 μM were obtained. This is

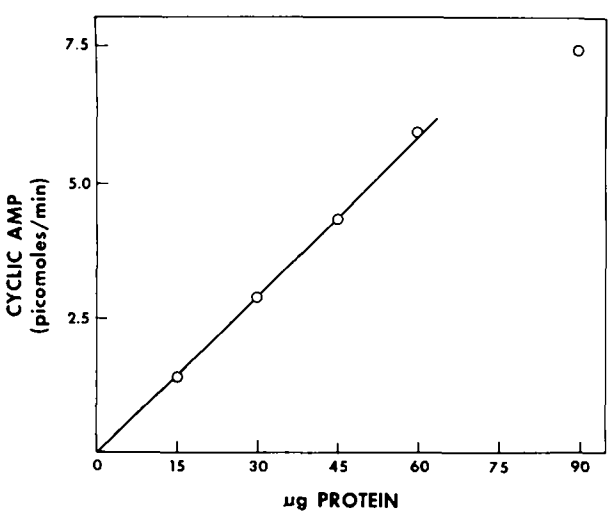

Fig. 2. Effect of protein concentration on adenylate cyclase reaction.

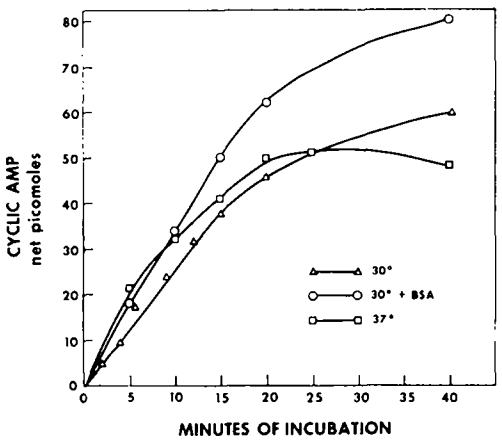

Fig. 3. Time course of adenylate cyclase reaction. AMP-PNP concentration was 0.5 mM.

slightly less than the values of 100–200 μM obtained by others in rat brain and other tissues [10, 11]. Since AMP-PNP is not depleted appreciably during the course of the reaction (see below), more satisfactory kinetic analysis is possible as shown in Fig. 4, where a K_m of 30–40 μM and V of approx. 130 pmoles/min per mg protein are obtained. While V with AMP-PNP is thus somewhat reduced, the K_m is favorable and the activity obtained is more than adequate.

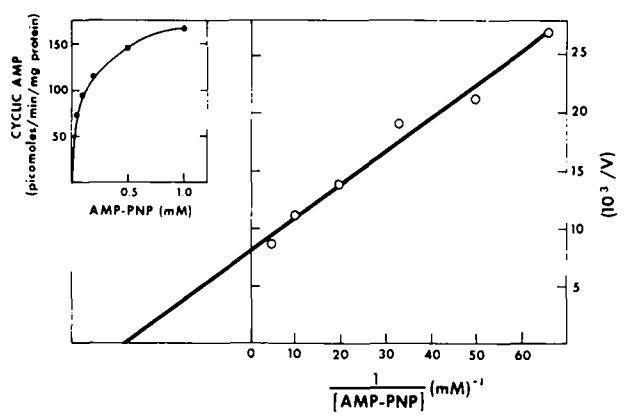

Fig. 4. Effect of AMP-PNP concentration on rate of cyclic AMP synthesis.

Addition of 10 mM NaF to the reaction mixture resulted in about a 2-fold activation of the cyclase, in agreement with the results of Perkins and Moore [10]. Stimulation by Mg^{2+} in the presence of 0.5 mM AMP-PNP was maximal at about 2 mM Mg^{2+} and higher concentrations (2–8 mM) did not significantly affect the cyclase (data not shown). Theophylline had a maximal beneficial effect at 0.5–2 mM. Higher concentrations were avoided since theophylline has been reported to inhibit adenylate cyclase activity [12]. When used methylisobutylxanthine was 0.1–0.2 mM, and gave essentially the same results as did theophylline.

AMP–PNP does not appear to be degraded significantly during the course of the cyclase incubation at 30 °C. Samples that had been allowed to incubate for 30 min at 30 °C in the presence of 30 μg of the cortical cyclase preparation were immediately chromatographed on Whatman No. 31ET paper and were found to contain 95–100% of the original AMP–PNP.

Cyclic AMP stability

The stability of the cyclic AMP produced was investigated under various storage conditions after the cyclase reaction was terminated by addition of acetate buffer. No apparent gain or loss of cyclic AMP was demonstrable when the samples were stored at either −20 °C or −90 °C for up to 7 days. In addition, during the assay of cyclic AMP, samples have remained at 0–3 °C for several hours without apparent change of cyclic AMP concentration.

Enzymatic degradation of cyclic AMP does occur during the course of the adenylate cyclase reaction (Table I). In the presence of 1 mM theophylline, approximately one-third of the added cyclic AMP was hydrolyzed in 10 min. The phospho-

TABLE I

EFFECT OF PHOSPHODIESTERASE INHIBITORS ON THE STABILITY OF CYCLIC AMP

10 pmoles of cyclic [^{3}H]AMP were added to triplicate samples containing the cyclase incubation mixture minus AMP–PNP plus inhibitors as shown. At zero time, 30 μg of the rat cortical cyclase preparation was added and the mixture incubated 10 min at 30 °C. The reaction was stopped with 1.0 ml HCl, pH 2.0 (final pH 3.5), containing 8.5 absorbance units unlabeled cyclic AMP. The mixture was added to a 4.0 cm × 0.6 cm Dowex-1-Cl column and washed with 6 ml of HCl, pH 2.6. Cyclic AMP was eluted with 3.0 ml HCl, pH 1.5. After lyophilization, a portion of each sample was counted for determination of cyclic AMP hydrolysis. This value was corrected for recovery of cyclic AMP as estimated by absorbance at 259 nm.

Inhibitor	% of cyclic [^{3}H]AMP remaining after cyclase incubation
None	10
0.1 mM theophylline	26
1.0 mM theophylline	66
0.1 mM RO 20-1724*	24
0.1 mM methylisobutylxanthine*	91
0.1 mM SQ 20009	76
1.0 mM SQ 20009	86
Control (minus enzyme)	100

* The solubility of RO 20-1724 and methylisobutylxanthine prevents the use of 1.0 mM solutions, although higher concentrations than those shown are feasible.

diesterase inhibitors SQ 20009 [13] and 1-methyl-3-isobutylxanthine [14] were more effective, with 90% of the added cyclic AMP remaining after 10 min incubation. RO 20-1724, although a potent inhibitor of cyclic AMP hydrolysis in rat erythrocytes [15], is no more effective under these conditions than equal concentrations of theophylline on rat cortical phosphodiesterase activity. It should be noted that the experiment outlined in Table I is not an assay for phosphodiesterase but rather a measurement of cyclic AMP hydrolysis under conditions of the adenylate cyclase reaction.

Further, it must be remembered that not only do some phosphodiesterase inhibitors exhibit tissue and species specificity to a high degree [15, 16], but that multiple phosphodiesterase activities from the same tissue have been described [17]. Adaptation of this assay to tissues other than rat cerebral cortex must be adequately controlled for species and tissue variations in the activity of phosphodiesterase. We routinely use theophylline or more recently 1-methyl-3-isobutylxanthine as standard inhibitors because of commercial availability and their potency in a wide variety of species and tissues. Although some cyclic AMP degradation was apparent in the presence of these inhibitors, assay of cyclase from sample to sample and from week to week was highly reproducible. This reproducibility and the enzyme rates obtained (see below) would seem to obviate the need for a cold cyclic AMP "trap" used in many other adenylate cyclase assays.

Assay of cyclic AMP

Simple means were devised for the introduction of the adenylate cyclase reaction mixture into the cyclic AMP protein binding assay. Attention was focused on methods for terminating the reaction and on possible interfering materials. Fig. 5 shows inhibition of the binding assay by AMP–PNP in the cyclase incubation mixture,

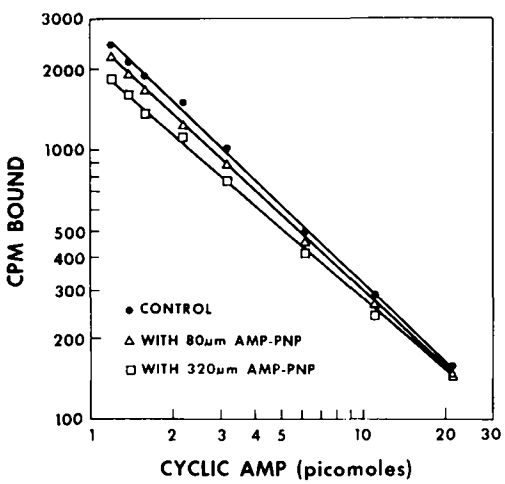

Fig. 5. Inhibition of cyclic AMP binding by AMP–PNP. Adenylate cyclase reaction mixtures (see Methods) containing 2.0 mM or 0.5 mM AMP–PNP in 100 μl were diluted with 150 μl of 83 mM sodium acetate, pH 3.9. The diluted reaction mixture (20 μl) was added to the cyclic AMP binding assay (final volume 50 μl) to give AMP–PNP concentrations of 320 μM and 80 μM in the binding assay. Cyclase reaction mixtures containing 40 μM AMP–PNP or less did not inhibit the binding assay. The cyclic AMP content indicated on the abscissa includes 1.2 pmoles cyclic [^{3}H]AMP and standard amounts of unlabeled cyclic AMP (0.2–20 pmoles).

at final concentrations of 320 μM or 80 μM. At the concentrations normally used, other components of the cyclase incubation mixture do not inhibit the binding reaction. Dilution of the reaction mixture to contain less than 40 μM AMP–PNP (final concentration in the binding assay) relieves all inhibition of the binding reaction. To avoid or compensate for inhibition of the binding assay at higher AMP–PNP concentrations,

we investigated several procedures to eliminate this error, which can be significant when adenylate cyclase activity is low.

If more than 20 pmoles of cyclic AMP are produced, it is possible to dilute samples sufficiently (e.g. to 500 μl) to eliminate all significant error due to inhibition of [^{3}H]AMP cyclic binding by AMP–PNP. If this is not possible, the inhibition may be compensated for by adding an appropriate concentration of AMP–PNP in the cyclase incubation mixture to the cyclic AMP solutions used for the binding assay standard curve (Fig. 5, Table II). Although the binding reaction is inhibited slightly, the AMP–PNP concentration in the standard curve and in the samples are equal and significant error is not introduced. This is true since substrate concentration does not change significantly during reaction. This condition is not met when ATP is the substrate.

TABLE II

CONTROLS FOR INHIBITION OF CYCLIC AMP BINDING ASSAY

Reaction mixtures (without protein) containing 2.0 mM or 0.5 mM AMP–PNP in 100 μl were incubated for 10 min at 30 °C, after which acetate (150 μl or 500 μl) was added. Cyclic AMP (10 pmoles) was added to one-half of these samples. Aliquots (20 μl) were assayed (final assay volume 50 μl), resulting in the final concentrations of AMP–PNP shown (320 μM, 80 μM, 33 μM). Other samples (2.0 mM AMP–PNP) were treated with HCl as described in methods for 30 min at 90 °C, lyophilized, dissolved in 50 mM sodium acetate, pH 4.5 (250 μl), and assayed. Additional samples were chromatographed on Dowex -1-Cl(see Table I) after addition of 0.3 pmole cyclic [^{3}H]AMP to assess recoveries during purification. The number of samples is shown in parentheses. Values represent apparent cyclic AMP in the total reaction mixture in pmoles ± S.E. except for $n = 2$, where the values are pmoles ± range.

	Control (blank)	Control + 10 pmoles cyclic AMP
I. Assayed without purification		
(a) 320 μM AMP–PNP	5.2 ± 0.3 (6)	16.5 ± 0.2 (2)
(b) 80 μM AMP–PNP	1.4 ± 0.2 (6)	13.1 ± 0.3 (2)
(c) 33 μM AMP–PNP	0.1 ± 0.2 (6)	9.9 ± 0.1 (2)
(d) 80 μM AMP–PNP with 80 μM AMP–PNP in cyclic AMP standard curve	−0.1 ± 0.1 (6)	10.1 ± 0.2 (2)
II. Assayed with purification		
(a) Acid hydrolysis (initially 320 μM AMP–PNP, see above)	2.6 ± 0.3 (4)	12.3 ± 0.3 (4)
(b) Dowex-1-Cl column (initially 80 μM AMP–PNP)	5.7 ± 0.7 (4)	15.2 ± 0.6 (4)

It is also possible to remove AMP–PNP without chromatography. Instead of stopping the reaction with acetate buffer, 50 μl of 0.3 M HCl is added (final concentration, 0.1 M), and the samples are heated at 90 °C for 30–60 min. The half-life of AMP–PNP is 15 min under these conditions. There is no detectable hydrolysis of cyclic AMP after 60 min at 90 °C. Under these conditions, the degradation products observed for AMP–PNP appeared to be ADP, AMP and adenine. It should be noted that the hydrolytic conditions (0.1 M HCl, 30–60 min, 90 °C) must be controlled, since cyclic AMP is hydrolyzed under more acidic conditions; e.g. at 100 °C in 1 M HCl its half-life is about 30 min [18]. After acid hydrolysis of AMP–PNP, the sample is lyophilized and dissolved in 50 mM sodium acetate, pH 4.5, for assay of cyclic AMP.

Samples in which the protein to be assayed is added after addition of pH 3.9 acetate or 0.3 M HCl serve as control blanks and usually give a blank activity of about 1–2 pmoles/min per mg protein when the rat cortical preparation is used. Some of this blank appears to be endogenous cyclic AMP since the blank can be lowered by inclusion of a second washing step in the centrifugation procedure used to prepare the crude adenylate cyclase.

Table II presents data demonstrating the efficacy of the procedures discussed above. Exogenous cyclic AMP was added to control samples to demonstrate recovery of cyclic AMP under differing conditions. Some samples were passed over Dowex-1-Cl columns to purify cyclic AMP before assay. It may be seen that high AMP–PNP concentrations interfere slightly with the binding assay but that dilution (e.g. to 33 μM AMP–PNP) or acid hydrolysis significantly improve binding accuracy and reproducibility.

Purification over Dowex columns is not only unnecessary when compared to hydrolysis or dilution but also can give an inordinately high blank. This is seen in Table II where the Dowex-1-Cl column fraction was dissolved in a small volume (250 μl) to assess the blank. The column procedure can still be of value, however, if large quantities of material must be removed. The blank can then be reduced by increasing sample resuspension volume and by more careful washing of the resin.

DISCUSSION

The assay method for adenylate cyclase described herein has several distinct advantages over previously published methods [1, 2, 7]. 1. It is very sensitive, both because the binding assay readily detects 0.1 pmole/aliquot and the blank values are either low or negligible. Less than 5 μg of protein can thus be accurately and reproducibly assayed. 2. The specificity of the detection system is high [5]. 3. The use of a substrate that cannot be cleaved at the β,γ-position insures reproducible kinetics and essentially constant substrate concentrations without the need for ATP regenerating systems that can be complicating factors. 4. The assay is relatively inexpensive, requiring only small quantities of AMP–PNP and cyclic [^{3}H]AMP, while other procedures may require μCi/tube quantities of labeled ATP or AMP–PNP. Further, this procedure obviates the need to utilize a short-lived, high-energy isotope.

There are two possible disadvantages of the system. First, a cold cyclic AMP "trap" cannot be utilized to protect labeled product. This matter has been discussed above and does not appear to be a major problem. Secondly, product detection by protein binding is slightly more time consuming than Dowex chromatography and Ba–Zn precipitation required with isotopically labeled substrate [1–3]. However, this "disadvantage" is, we feel, more than offset by the utility of a single, sensitive and specific method applicable to both assay of cyclic AMP and adenylate cyclase and by the fact that this means of analysis is the source of the majority of the advantages described.

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